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Low-Energy Diode Laser Irradiation Reduced Plasminogen Activator Activity in Human Periodontal Ligament Cells

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Background and Objective: **The plasminogen activator (PA)-plasmin proteolytic system is implicated in the degradation of the extracellular matrix in inflammation. Since human periodontal ligament (PDL) cells produced high PA activity in response to mechanical stress, excessive mechanical stress to PDL cells such as occlusal trauma may induce collagen breakdown through activation of the PA-plasmin system. As low-energy laser irradiation has anti-inflammatory effects, we examined the effects of low-energy laser irradiation on the PA-plasmin system in stretched PDL cells in vitro.**
Study Design/Materials and Methods: **Human PDL cells obtained from healthy premolars were mechanically stretched and Ga-Al-As low-energy laser was irradiated (830nm, 3.95 to 7.90 J/cm^2) to the stretched cells.**
Results: **PDL cells showed a marked elevation in PA activity in response to stretching, which was significantly inhibited by a laser irradiation in a dose-dependent manner (55–86%, $p < 0.001$). This effect could involve transcriptional events of tissue type (t) PA gene.**
Conclusion: **These results suggests that laser irradiation may reduce collagen breakdown around the PDL associated with traumatic occlusion. Lasers Surg. Med. 21:456–463, 1997.**

Key words: Ga-Al-As diode laser; mechanical stress; periodontal ligament; plasminogen activator-plasmin system

INTRODUCTION

The periodontal ligament (PDL) is a dense connective tissue, surrounding the dental root and connecting it with alveolar bone. The most important elements of PDL are the collagenous principal fibers, with the terminal portions of the fibers inserted into two hard tissues, cementum and alveolar bone. PDL therefore, functions as a tooth-supporting tissue and also a cushion between the two hard tissues to mitigate the occlusal force such as compression or tension. When occlusal forces exceed the adaptive capacity of the tissues, the tissues are injured and occlusal trauma will occur. An excessive mechanical force such as occlusal trauma is associated with alveolar bone loss in severe periodontitis [1–3], which may be caused by some factors associated with periodontal tissue breakdown.

Plasmin, the active protease which is converted from plasminogen by plasminogen activator (PA), is capable of activating latent collagenase. Plasminogen, found in abundance in the ex-

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tracellular matrix, and control of the conversion of plasminogen into plasmin, is regulated by the activity of PA secreted from various cells. Therefore, PA is considered to play a central role in the breakdown of the extracellular matrix during tissue destruction [4–6]. PA activity is also controlled by the PA inhibitor (PAI), PAI-1, which rapidly inhibits the activity of PA [7] and has been detected in a variety of cell types [8,9]. Since the level of activity of PA and plasmin are increased in the gingival fluid in periodontal disease [10] and plasmin activity was shown to be markedly decreased by periodontal treatment [11], the PA-plasmin system of PDL cells may affect the tissue destruction associated with periodontal disease. We reported that PDL cells showed marked elevation in PA activity in response to mechanical stretching and the association of PA and PAI activities with the tissue degradation induced by mechanical stress [12].

Among the many physiological effects of low-energy laser irradiation, anti-inflammatory effects have been reported [13–18]. We also demonstrated that low-energy laser irradiation significantly inhibited prostaglandin (PG) E_2 and interleukin (IL)-1β production from PDL cells subjected to mechanical stress [19], with the resulting anti-inflammatory effect.

The purpose of the present study was to determine the effect of low-energy laser irradiation on the activity and gene expression of PA and PAI-1 from stretched human PDL cells.

MATERIALS AND METHODS

Cell Culture

PDL cells were prepared from premolars extracted from healthy young patients in the course of orthodontic treatment according to the method of our previous study [19] which is a modified method of Somerman et al. [20]. Informed consent was obtained from the patient after the nature of the study had been fully explained. The medium used was α-MEM (Gibco, Grand Island, NY) supplemented with 100μg/ml penicillin-G (Sigma Chemical Co., St. Louis, MO), 100μg/ml kanamycin sulfate (Meiji Seika Kaisha, LTD., Tokyo, Japan), 0.3μg/ml amphotericin B (Flow Laboratories, McLean, VA), and 10% fetal calf serum (FCS, Cell Culture Laboratories, Cleveland, OH); the cultures were kept at 37°C in a humidified incubator in the presence of 95% air and 5% CO_2. When the cells developing from the explants had reached confluence in 35-mm culture dishes (Falcon, Franklin Lakes NJ), they were subcultivated in culture flasks (Falcon, #3110) with 0.05% trypsin (Gibco) in PBS. Some cells still attached to the bottom of the flask were discarded during serial passage to avoid contamination by epithelial cells, which are less easily detachable than fibroblasts [21]. The cells were used at the 7th passage.

Application of Cyclic-tension Force

Cyclic-tension force was applied to PDL cells according to our previous study [19] with a Flexercell Strain Unit (Flexcell Corp. PA), the apparatus for which was originally developed by Banes et al. [22]. PDL cells (1×10^5/well) were seeded onto the flexible-bottom plates (FLEX I® culture plates, Flexcell Corp.) and cultured for 3 days until confluent. The medium was then replaced with the same medium as described above, except that it contained 2% FCS instead of 10%. After the cells were cultured for a further 24 hours, the medium was changed again, and then the cells attached to the flexible-bottom were elongated. PDL cells were subjected to 18% elongation by the Strain Unit at 6 cycles/min (i.e., 5-sec elongation and 5-sec relaxation) for the 1–5 days of this experiment according to the protocol of our previous study on the detection of PGE_2 [23] and IL-1β [19]. As the nonelongation control, the cells were seeded onto the nondeformable surfaces (FLEX II® culture plates, Flexcell Corp.) made with the same substrate material present in the flexible-bottom plates, and the plates were then placed into the Strain Unit.

Laser Irradiation

The Ga-Al-As diode laser (model Panalas-1000, Matsushita, Inc., Tokyo, Japan), which has a continuous wavelength of 830 nm and a maximum power out put of 700 mW, was used in this study. The laser beam delivered by an optical fiber 0.6-mm in diameter was expanded at the tip of the fiber and irradiated a circular area, 130mm in diameter at the cell-layer level. The power density of the laser beam in the center area (65mm in diameter) of the irradiation circle was uniform measured by using a laser power meter. In this manner, the 4 wells out of 6 well plates were simultaneously and uniformly irradiated within the center area. The time of exposure was 10 and 20 min per day, and the total energy corresponding to 10–20-min exposures, varied from 3.95–7.90 J/cm^2 (19.4–38.8 J/well) which dose is similar to that used in our previous study [24]. Irradia-

tion was performed once a day for 1,3, and 5 days to the cells with or without application of tension force. During the 5-day experimental period, the medium was not changed; the conditioned medium from each well was collected on days 1,3, and 5, and was assayed for PA and PAI activities. After each treatment, the cells were washed twice with PBS and detached with trypsin (Gibco); cell numbers per well were then counted with a Coulter Counter (Model ZM, Electronics Ltd., Northwell Drive, Luton, Beds, England).

PA and PAI Assay

PA activity in the conditioned PDL cell medium was measured according to the method of Pfeilshifter et al. [25]. Conditioned medium (30μl), 6μl plasminogen (10 unit/ml; Kabi Vitrum Stockholm, Sweden), and 12μl plasmin substrate S2251 (1.8 mM; Kabi Vitrum) and 3μl PA stimulator (3 mg/ml;Kabi Vitrum) were added to 96-well plates, and 0.05M Tris buffer (pH 7.4) was added to a final volume of 75μl. The assay reaction was stopped by 100μl of 20 mg/ml citric acid. The chromogenic substrate, S2251, was hydrolyzed by plasmin which was converted from plasminogen by PA in the conditioned medium from PDL cells. Color development was followed at 415 nm in a microplate reader (MTP-32, Corona, Ibaraki, Japan). The reaction mixture without plasminogen and PA stimulator served as the assay control. The PA activity was calculated per 10^5 cells, determined from the *p*-nitroaniline (*p*-NA) standard curve, with 1 unit representing the release of 1μmol *p*-NA/min.

To examine whether the alteration of PA activity is due to the alteration of PA itself and/or PAI, we determined the PAI-1 level in the conditioned medium of PDL cells using an ELISA kit for PAI-1 assay (IMULYSE™ PAI-1, Biopool AB, Umea, Sweden).

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA of PDL cells subjected to tension force and laser irradiation for 3 days was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [26]. The final RNA precipitate was stored in ethanol at −135°C. cDNA synthesis and amplification by RT-PCR were carried out using GeneAmp RNA kit (Perkin-Elmer, New Jersey, U.S.A.). Briefly, cDNA synthesis was carried out at 42°C for 15 min in 20μl final volume containing 4 μl of $MgCl_2$ (25mM), 2 μl of 10× PCR Buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2 μl of dNTP (10 mM), 1 μl of RNase Inhibitor (20 U/μl), 1 μl of MuLV reverse transcriptase (25 U/μl), 1 μl of random hexamer (25 mM), 1 μl of oligo $d(T)_{16}$(25μM), and 2 μl of total RNA (1μg/μl). The PCR mixture containing 20 μl of the cDNA solution, 4 μl of 25 mM $MgCl_2$, 8 μl of 10× buffer II, 1μl of forward primer, 1 μl of reverse primer, 65.5 μl of H_2O and 0.5 μl of AmpliTaq DNA polymerase was subjected to amplification using a Gene Amp PCR system 9600 (Perkin-Elmer) set at 94°C for 1 min, 55°C for 2 min and 72°C for 2 min for 21–36 cycles.

The primers for tissue type (t) PA, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Biologica Co. (Nagoya, Japan). The PCR primers for amplification were designed referring to the sequences of cDNA that had reported for tPA [27], PAI-1 [28] and GAPDH [29].

The following primer sequences with the size of the amplified fragment in brackets were used: tPA [946bp], 5′-CAG GAA ATC CAT GCC CGA TT-3′ and 5′-GCT GAT GAG TAT GCC CCC GCA CAG GAA CCG-3′, PAI-1 [314bp], 5′-GGA TCC AGC CAC TGG AAA GGC AAC ATG-3′ and 5′-GGA TCC GTG CCG GAC CAC AAA GAG GAA-3′, GAPDH [318bp], 5′-ATC ACC ATC TTC CAG GAG-3′ and 5′-CTC ATG ACC ACA GTC CCA T-3′.

To evaluate saturated or incomplete reaction of PCR, three gene fragments were amplified simultaneously every 3 cycles from the 21st–36th cycle. PCR fragments amplified by different cycles were separated by electrophoresis on 1.5% agarose gel, and subsequently stained with ethidium bromide. The relative intensities were quantitated by image analyzer (ATTO densitograph, ATTO Corp., Tokyo, Japan).

Statistical Method

Values were calculated as the mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA) as indicated in the results. Student's *t*-test was used for analysis of the difference in some groups.

RESULTS

Effect of Tension Force and Laser Irradiation on Cellular Proliferation

Cell counts in each well demonstrated that there were no significant differences among the

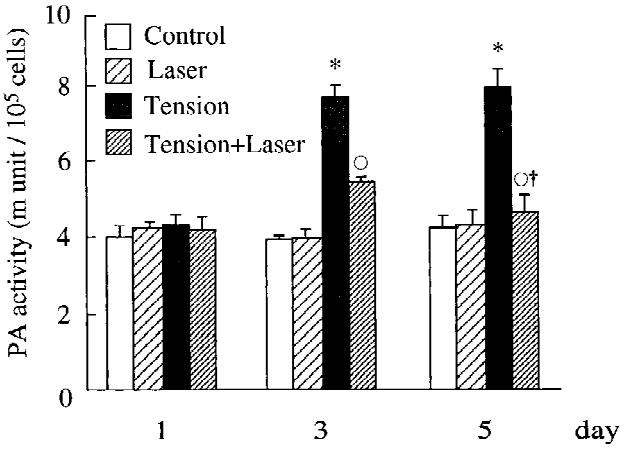

Fig. 1. Effects of the duration of tension force and laser irradiation (10min/day) on PA activity by human PDL cells. Laser irradiation for 10 min/day for 3 and 5 days significantly inhibited PA activity stimulated by tension force.
Values are mean ± SD for 4 cultures. *Significantly different from corresponding control ($p < 0.001$). °Significantly different from corresponding tension ($p < 0.001$). †Significantly different from corresponding 3 days ($p < 0.05$). The experiment was repeated 3 times from 3 different cells with similar results.

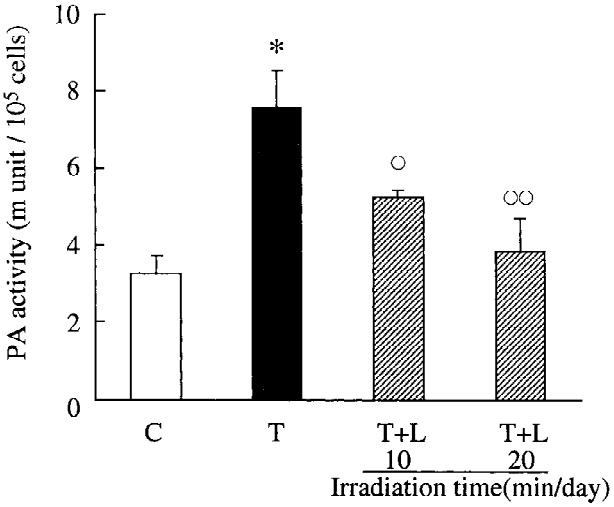

Fig. 2. Effects of tension force and different doses of laser irradiation for 3 days on PA activity. Laser irradiation for 10 and 20 min/day significantly inhibited PA activity stimulated by tension force in a laser dose-dependent manner (by one way-ANOVA, $p < 0.001$). Values are mean ± SD for 4 cultures. *Significantly different from control ($p < 0.001$). Significantly different from tension ($^{\circ}p < 0.01$, $^{\circ\circ}p < 0.001$). C: Control, T: Tension, T + L: Tension plus laser. The experiment was repeated twice from 2 different cells with similar results.

four groups which were Control, Laser, Tension and Tension-plus-Laser, at various time periods (data not shown). Furthermore, laser irradiation under the present experimental conditions did not induce temperature rise in the media (data not shown).

Effect of Laser Irradiation on PA Activity

The activity of PA by human PDL cells in response to a mechanical tension force of 18% elongation applied by the Flexercell Strain Unit and the inhibitory effect of laser irradiation (10min/day) are shown in Fig. 1. Control PDL cells constitutively synthesized some amount of PA without the application of tension force; neither tension force nor one-day laser irradiation to the cells affected PA activity. However, 3 and 5 day application of tension force produced a similar significant increase in PA activity, which was about two-fold higher than that of the controls ($p < 0.001$). Furthermore, laser irradiation of the cells for 3 days significantly inhibited PA activity (58%, $p < 0.001$) stimulated by the tension force, and irradiation for 5 days inhibited 89% of stimulated PA activity ($p < 0.001$). Inhibitory effect in 5 days irradiation group was higher than that in 3 days group ($p < 0.05$). Laser irradiation did not affect the level of PA activity of the control on days 3 and 5.

The effects of different irradiation doses on PA activity in stretched PDL cells are shown in Fig. 2. The cells subjected to tension force were irradiated for 10 or 20 min/day for 3 days. Daily irradiation for 10 min (T + L, 10min) markedly inhibited PA activity (55%, $p < 0.01$) and irradiation for 20 min (T + L, 20min) further inhibited PA activity (86%, $p < 0.001$) compared to the non-irradiated tension (T). The inhibitory effect was laser dose-dependent as determined by one-way ANOVA ($p < 0.001$).

Effect of Laser Irradiation on PAI-1 Activity

The activities of PAI-1 by human PDL cells in response to a mechanical tension force and laser irradiation are shown in Fig. 3. Although PDL cells constitutively produced some amount of PAI-1 activity which increased in a time-dependent manner (by one-way ANOVA, $p < 0.001$) in all four groups, neither tension force nor laser irradiation to the cells affected PAI-1 activity over the experimental period.

Effect of Laser Irradiation on Gene Expression

To elucidate the molecular mechanisms of alteration of PA activity by laser irradiation, tPA and PAI-1 mRNA expression in both the control

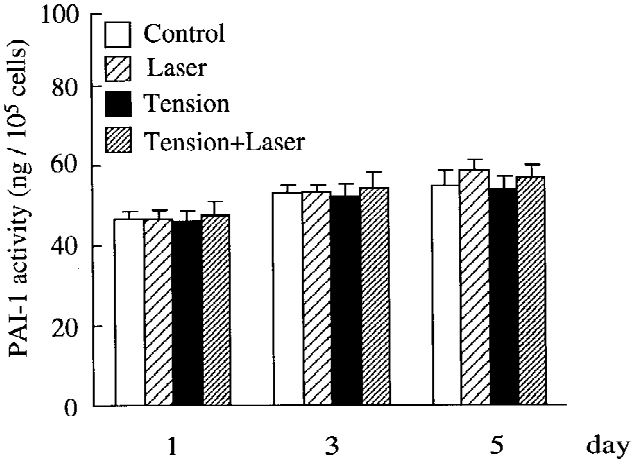

Fig. 3. Effects of duration of tension force and laser irradiation (10min/day) on PAI-1 activities by human PDL cells. There were no significant differences among the four groups at various time periods, although PDL cells constitutively demonstrated PAI-1 activity which increased in a time-dependent manner (by one-way ANOVA, $p < 0.001$). Values are mean ± SD for 4 cultures. The experiment was repeated twice from 2 different cells with similar results.

and laser irradiated cells were investigated by RT-PCR analysis. As PA activity was increased by 3-day application of the tension to the cells, gene expression was investigated on day 3 (Fig. 4).

Each band of the three gene fragments amplified simultaneously every 3 cycles constantly increased as the number of cycles increased, suggesting that PCR reactions would be neither saturated nor incomplete. The bands for tPA mRNA of PDL cells were visible on the 30th cycle, with the bands of the stretched cells being more intense than those for the corresponding unstretched controls. However, the bands for tPA mRNA became much less intense by laser irradiation. Neither tension force nor laser irradiation to the cells affected the bands for PAI-1 mRNA.

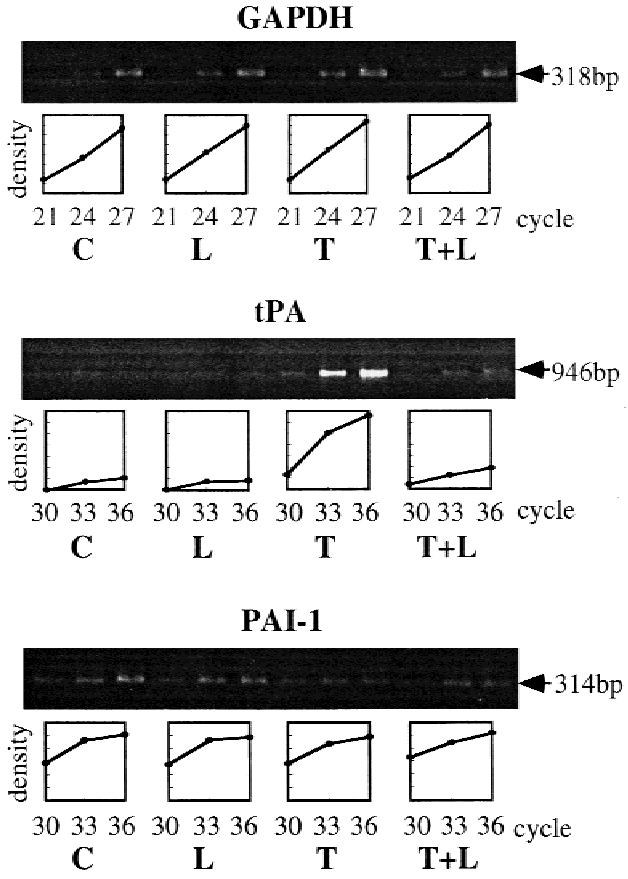

Fig. 4. Ethidium bromide staining pattern of simultaneously amplified PCR products on agarose-gel electrophoresis. Total RNA was extracted from the Control (C), Laser (L), Tension (T) and Tension plus Laser (T + L) PDL cells, and 2μg of total RNA was reverse-transcripted; 1/10 of the cDNA obtained was amplified for each mRNA to obtain DNA fragments of the size indicated. The gene expression of tPA was markedly increased in PDL cells subjected to tension force and it was significantly inhibited by laser irradiation. However, the gene expression of PAI-1 was not affected by either the tension force or laser irradiation. The figures represent the results from one of two similar experiments from 2 different cells.

DISCUSSION

We previously reported that application of tension force induced a marked elevation of PA activity from the PDL cells in a time- and magnitude-dependent manner, but tension force did not affect PAI-1 activity, and we discussed an important role of PA activity on the degradation of periodontal tissue by mechanical stress such as occlusal trauma [12]. Because low-energy laser irradiation is used as an anti-inflammatory [13–18], we examined the effects of laser irradiation on the PA-plasmin system.

In the present study, the findings provide the first evidence that low-energy laser irradiation significantly inhibits the increased PA activity induced in human PDL cells in response to a mechanical tension force. This inhibition was not due to the number of cells being reduced by laser irradiation. The laser-induced inhibitory effects on PA activity were time-dependent, with almost complete inhibition found in the 10-min irradiation group on day 5, with inhibitory effects also being laser-dose dependent. Since PAI-1 inhibits PA activity, the modulation of PA activity by PAI-1 production is an important factor in the regulation of the net effect of the PA-plasmin system. In the present study, PDL cells constitu-

tively synthesized some amount of PAI-1, neither tension force nor laser irradiation to the cells affected PAI-1 activity over the experimental period. It suggests that stimulation and inhibition of PA activity was due to PA itself but not PAI-1.

The two types of human PA which have been identified in previous studies, uPA and tPA, can be distinguished by differences in molecular weight, affinity for fibrin, and immunoreactivity [4,30]. Their difference in properties and tissue distribution suggest that they may have different functions. uPA, is thought to be involved in more generalized proteolysis and has increasingly been implicated in tumor invasion [31]. In contrast, tPA, which is activated by fibrin, is thought to be a key enzyme involved in fibrinolysis [30]. However, our previous study showed that tension force stimulated the protein and gene expression of tPA in PDL cells but not those of uPA [12]. Therefore, the effect of laser on tPA-gene expression was examined in the present study. Significant stimulation of tPA-gene expression by tension force for 3 days was significantly inhibited close to the control level by laser irradiation. However, PAI-1-gene expression was not affected. These phenomena can be comparable with the phenotypic expression of these factors. Diamond et al. [32,33] and Iba and Sumpio [34] reported that cyclic strain stimulated tPA protein and gene expression, but not those of PAI-1 [12]. It may be possible that mechanical stress may affect only tPA expression but not that of uPA and PAI. Since the tension force and laser irradiation affect the same factors of the PA-plasmin system, similar mechanisms or mediators seem to be involved in the stimulation by tension and inhibition by laser irradiation.

Although the mechanism responsible for the transduction of mechanical stimuli to biochemical signals is not yet clear, a number of such mechanisms have been suggested: Elevated intercellular calcium levels are required for the acute release of tPA [36]. The production of diacylglycelol (DAG) and protein kinase C regulates the synthesis of tPA [37]. Cyclic strain enhanced phosphatidylinositol turnover with the production of inositol trisphosphate (IP_3) and DAG [38] and activation of protein kinase C [39] within seconds of the initiation of cyclic strain. Using the same experimental model as the present one, we previously reported that mechanical tension force stimulated IP_3 production, the level of which was maximal on day 1 and decreased on days 3 and 5 in PDL cells [23]. Since PA activity increased on day 3 and 5 but not on day 1 in the present experiment, as well as the time-lag of production of PA and IP_3, involvement of the IP_3 mechanism in PA activity of PDL cells by tension and laser irradiation may be indirect or weak.

Considering the factors mediating PA activities, some mediators are reported to stimulate PA activity. It is reported that basic fibroblast growth factor increased uPA activity [39,40]. In contrast, vascular endothelial growth factor and vascular permeability factor increased tPA activity [41]. In addition, epithelial growth factor and transforming growth factor-α also increased tPA activity [42] in endothelial cells. Furthermore, parathyroid hormone, 1,25-dihydroxyvitamin D_3, PGE_2, epithelial growth factor, tumor necrosis factor and IL-1β [43–45] increased tPA activity in osteoblast-like cells. Among these factors, PGE_2 is one of the most important induced by mechanical stimuli [24,46] and IL-1β is also produced by mechanical stress [19]. Interestingly, we recently reported that low-energy laser irradiation completely inhibited PGE_2 production and partly inhibited IL-1β production (40%) which had been stimulated by mechanical stretching [24].

These results strongly led us to speculate that PGE_2 and/or IL-1β may, at least, partly mediate PA activity stimulated by mechanical tension and inhibited by low-energy laser irradiation. Further studies on the effect of the inhibition of endogenous PGE_2 and IL-1β production on PA activity and the effect of exogenous application of these factors during laser irradiation on PA activity may elucidate the roles of these factors as the mediators of PA activity by mechanical tension and laser irradiation.

It is reported that excessive mechanical force such as occlusal trauma is associated with alveolar bone loss in severe periodontitis [1,2]. Recently, Jin and Cao [3] reported, based on substantiated evidence rather than clinical impression, that teeth with occlusal trauma had less osseous support than teeth without trauma. Linde [47] also reported that trauma of occlusion may enhance the rate of progression of plaque-associated periodontal disease. Furthermore, there are some studies suggesting some relationship between periodontal disease and the PA-plasmin system. Hidaka et al. [10] reported that the activity of plasmin and PA are increased in the gingival fluid in periodontal disease, with plasmin activity being markedly decreased by periodontal treatment [11]. Such evidence suggests that the alteration of the PA-plasmin system

may affect the progress of periodontal disease. Because low-energy laser irradiation markedly inhibited PA activity dose- and time-dependently in the present experiment, it may decrease collagen breakdown via lower PA activity around the PDL subjected to mechanical stress.

There are some anti-inflammatory effects of low-energy laser irradiation in vivo, including inhibition of carrageenin inflammation in rats [13,14], inflammatory cellular infiltration in the synovial membrane of a patient with rheumatoid arthritis [16,17], PGE_2 in synovial fluid of a patient with rheumatoid arthritis [18]. We also reported the inhibition of PGE_2 and IL-1β production from stretched human PDL cells by laser irradiation in vitro [24]. Inhibitory effect of low-energy laser irradiation on PA activity can be considered as anti-inflammatory, perhaps reflecting an in vivo event.

In conclusion, low-energy laser irradiation significantly inhibited PA activity in stretched PDL cells in vitro. This factor is involved in the tissue destruction though activation of collagenase, a thesis supported by the markedly high levels found in the gingival fluid in periodontal disease in vivo. Our findings indicate that laser irradiation may be of therapeutic benefit against the aggravation of periodontal disease accompanying traumatic occlusion. However, considering that approximately 25% of the diode laser beam (60mW) penetrates to a depth of 2.0mm in human mandibular cortical bone [48], a higher laser dose (4–5-fold) may be necessary for clinical use to reach enough energy to the PDL.

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